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Rapid Identification of Clinically Significant Species and Taxa of Aerobic Actinomycetes, Including *Actinomadura*, *Gordona*, *Nocardia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* Isolates, by DNA Amplification and Restriction Endonuclease Analysis

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A previously described PCR-restriction fragment length polymorphism (RFLP) identification schema for *Nocardia* that used an amplified 439-bp segment (amplicon) of the 65-kDa heat shock protein gene was evaluated for potential use with isolates of all clinically significant aerobic actinomycetes. The study included 28 reference (American Type Culture Collection) strains and 198 clinical isolates belonging to 20 taxonomic groups. Of these 198 isolates, 188 could be differentiated by this PCR-RFLP method. Amplicons from all aerobic actinomycete isolates lacked *Bst*EII recognition sites, thereby distinguishing them from those of mycobacteria that contain one or more such sites. Of 29 restriction endonucleases, *Msp*I plus *Hin*fI produced RFLP patterns that differentiated 16 of the 20 taxa. A single RFLP pattern was observed for 15 of 20 taxa that included 65% of phenotypically clustered isolates. Multiple patterns were seen with *Gordona bronchialis*, *Nocardia asteroides* complex type VI, *Nocardia otitidiscaviarum*, *Nocardia transvalensis*, and *Streptomyces* spp. *Streptomyces* RFLP patterns were the most heterogeneous (five patterns among 19 isolates), but exhibited a unique *Hin*fI fragment of >320 bp. RFLP patterns that matched those from type strains of *Streptomyces albus*, *Streptomyces griseus*, or *Streptomyces somaliensis* were obtained from 14 of 19 *Streptomyces* isolates. Only 10 of 28 isolates of *N. otitidiscaviarum* failed to yield satisfactory amplicons, while only 6 of 188 (3.2%) clinical isolates exhibited patterns that failed to match one of the 21 defined RFLP patterns. These studies extended the feasibility of using PCR-RFLP analysis as a rapid method for the identification of all clinically significant species and taxa of aerobic actinomycetes.

Traditional differentiation of genera and species comprising the aerobic actinomycetes has involved the examination of staining characteristics and colonial morphology and the evaluation of various biochemical reactions. These methods have not always been reproducible and have been inadequate for the identification of recently characterized *Nocardia* species (e.g., *Nocardia farcinica* and *Nocardia nova*), for which additional antibiogram and specialized biochemical data are required for differentiation (21, 28, 29, 32). All of these methods are time-consuming and laborious (2, 14, 16, 23).

The turnaround time for the identification of most species of slowly growing mycobacteria has been greatly shortened by the availability of high-performance liquid chromatography (HPLC) (3, 6, 11, 24), although it has not been generally useful for the separation of rapidly growing *Mycobacterium* species (5, 15, 24). HPLC also has been used routinely to differentiate the genera *Actinomadura* and *Streptomyces*, which lack mycolic acids, from mycolic acid-producing genera as well as to separate the genera *Corynebacterium*, *Gordona*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* (3, 4, 10). HPLC has shown some utility in the differentiation of some closely related species of *Nocardia* (28); however, identification to the species level within the aerobic actinomycetes is not possible for most species and is a major taxonomic limitation of this methodology (3, 4, 10, 28).

Use of PCR coupled with restriction endonuclease and/or probe hybridization analyses of PCR products has been the focus of recent interest for the separation of mycobacteria from the nocardiae (12, 17) as well as for the recognition of species within the genera *Mycobacterium* and *Nocardia* (1, 8, 9, 13, 14, 18, 21–23, 27, 28). This methodology has proven to be sensitive, less time-consuming, and less labor-intensive than traditional biochemical methods (14, 19, 22, 23, 28). While the differentiation of species within the genera *Mycobacterium* (1, 8, 9, 17, 20, 22, 23, 27) and *Nocardia* (14, 21, 28) by this technique has been described, to our knowledge, no molecular biological protocols for differentiating species or taxa of other clinically significant aerobic actinomycetes have been described.

This study was therefore undertaken to apply the PCR and restriction endonuclease analysis methodologies to an expanded population of isolates that included all the commonly encountered pathogenic species of aerobic actinomycetes other than those of mycobacteria for which PCR-based protocols have been described previously (22, 23).

MATERIALS AND METHODS

Organisms. The present study included 198 clinical isolates and 28 reference strains from the American Type Culture Collection (ATCC; the latter were kindly provided by P. Pienta, Rockville, Md.) comprising 18 taxonomic groups of aerobic actinomycetes (Table 1). These included 12 *Gordona bronchialis* isolates; 5 *Gordona sputi* isolates; 4 *Gordona* sp. isolates; 5 *Rhodococcus equi* isolates; 8

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TABLE 1. Clinical and reference isolates of aerobic actinomycetes used in the present study

Aerobic actinomycete species or taxa	No. of clinical isolates	Reference isolates (ATCC strain no.)
G. bronchialis	11	25592^T
G. sputi	3	29627^T, 33610
Gordona sp.	4	0
R. equi	4	6939^T
T. paurometabolum	6	8368^T, 25938
A. madurae	8	13723, 13724, 19425^T
Streptomyces sp.[a]	15	0
S. albus	0	3004^T
S. griseus	0	10137, 23345^T
S. somaliensis	0	33201^T
Nocardia asteroides complex		
Type I[b]	12	23824
Type II	7	0
Type IV	10	49872, 49873
Type VI	24	14759
N. brasiliensis	13	19296^T
N. farcinica	12	3308, 3318, 23825
N. nova	15	33726^T, 33727
N. otitidiscaviarum	27	14629^T
N. transvalensis	10	6865^T, 29982
N. pseudobrasiliensis[c]	17	51511, 51512^T
Total	198	28

[a] Identified to the genus level only.
[b] Antimicrobial susceptibility types described by Wallace et al. (31).
[c] Described by Wallace et al. (28) and Ruimy et al. (18).

Tsukamurella paurometabolum isolates; 11 *Actinomadura madurae* isolates; and 19 *Streptomyces* isolates, including *Streptomyces albus* ATCC 3004^T, *Streptomyces griseus* ATCC 10137 and ATCC 23345^T, and *Streptomyces somaliensis* ATCC 33201^T. The *Nocardia* isolates included for comparison involved 57 isolates of four *N. asteroides* complex antibiogram types (31), including 13 type I isolates, 7 type II isolates, 12 type IV isolates, and 25 type VI isolates. Additional *Nocardia* species included 14 *Nocardia brasiliensis* isolates, 15 *Nocardia farcinica* isolates (32), 17 *Nocardia nova* isolates (29), 28 *Nocardia otitidiscaviarum* (formerly *Nocardia caviae*) isolates, 12 *Nocardia transvalensis* isolates, and 19 *Nocardia pseudobrasiliensis* isolates (18, 28).

Clinical isolates of aerobic actinomycetes were chosen from isolates submitted to the Mycobacteria/Nocardia Research Laboratory at the University of Texas Health Center at Tyler, Tex. (UTHCT), for susceptibility testing and from isolates recovered from clinical samples at the Tuberculosis Section, State Health Laboratory, in Brisbane, Queensland, Australia. All clinical isolates used in this study were identified by previously described biochemical and antimicrobial susceptibility methods (2, 16, 21, 25, 26, 28–32). Clinical isolates listed as *Gordona* spp. failed to match the biochemical species patterns described by Tsukamura (26), while clinical isolates of *Streptomyces* were identified to the genus level only. The identities of many of the isolates were confirmed by the Texas State Department of Health, Austin; the Actinomycete Reference Laboratory, Centers for Disease Control and Prevention, Atlanta, Ga.; and the State Health Laboratory, Brisbane, Queensland, Australia. Isolates of *Gordona* and *Streptomyces* were identified to the genus level, and *R. equi*, *T. paurometabolum*, and selected *Nocardia* isolates were identified to the species level by HPLC as described previously (3, 4, 28).

PCR amplification. Actinomycete DNA was prepared by previously described methods (21). A 439-bp segment of the 65-kDa heat shock protein (HSP) gene (22, 23) was amplified from ground cell supernatants by PCR with 1.0 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, Ind.) in optimized buffer E (1.5 mM $MgCl_2$ [pH 9.0]; Invitrogen, San Diego, Calif.) containing 42 μM (each) deoxynucleoside triphosphate, 9% dimethyl sulfoxide, and 1 μM (each) primers TB11 (5′-ACCAACGATGGTGTGTCCAT) and TB12 (5′-CTT GTCGAACCGCATACCCT) (Midland Certified Reagent Co., Midland, Tex.), together with the appropriate positive and negative controls, by a modification of the method of Telenti et al. (23). The PCR mixtures were run for 45 cycles at 94, 55, and 72°C for 1 min each and then for a 10-min extension period at 72°C.

RFLP analysis. Commercially available restriction endonucleases (New England Biolabs, Beverly, Mass., and Promega, Madison, Wis.) were screened for optimal restriction fragment length polymorphism (RFLP) band patterns by incubation at the appropriate temperatures and by using buffers at the 10× concentration recommended by the manufacturers, with the exception of *Bsa*HI. To achieve complete digestion with *Bsa*HI, acetylated bovine serum albumin was substituted for bovine serum albumin and the digestion mixture was incubated at 60°C for 1 h.

Restriction fragments were electrophoresed on 3% Metaphor agarose (4-bp resolution; FMC Bioproducts, Rockland, Maine) containing ethidium bromide (0.625 μg/ml) in a Mini-Sub-Cell electrophoresis system (Bio-Rad, Richmond, Calif.) at 95 V for 1.5 to 2.0 h. Fragment sizes (in base pairs) were estimated on a computerized Bio Image system (Millipore, Bedford, Mass.), with a 100-bp ladder (Life Technologies, Grand Island, N.Y.) and a pGEM-bp ladder (Promega) used as molecular size standards. Fragments of ≤60 bp were disregarded as PCR primer artifacts, as discussed by Telenti et al. (23).

RESULTS

PCR amplification. Satisfactory amplicons were readily obtained from all test isolates except those of *N. otitidiscaviarum*, of which only 18 of 28 isolates produced satisfactory amplicons, as noted previously (21).

RFLP analysis. In a prior report (21), 12 species and taxa of *Nocardia* were successfully differentiated by PCR and restriction endonuclease analysis with *Msp*I and *Bsa*HI. However, these two enzymes failed to differentiate 6 of the 18 species and taxa of aerobic actinomycetes under consideration in this study, namely, (i) 2 of 12 isolates of *G. bronchialis*, all 12 isolates of the *N. asteroides* complex type IV, and 7 of 12 isolates of *N. transvalensis*, (ii) all isolates of *N. brasiliensis* and *R. equi*, and (iii) all isolates of *G. sputi* and *G. bronchialis.*

Screening of 29 restriction endonucleases resulted in the selection of *Msp*I and *Hin*fI as the optimal endonucleases for amplicon digestion, followed by digestion with *Bsa*HI as a secondary enzyme for use in instances in which species differentiation was not complete. *Msp*I and *Hin*fI produced 19 RFLP band patterns that successfully differentiated 14 of 18 clinical taxa. One of the two remaining paired taxa, *N. brasiliensis* and *N. otitidiscaviarum*, was differentiated by *Bsa*HI, as reported previously (21) (Fig. 1). As reported in the prior study (21), the remaining two clinically rare taxa, *N. asteroides* complex type IV isolates and 7 of 12 *N. transvalensis* isolates, could not be differentiated further with *Bsa*HI.

The remaining 26 restriction endonucleases produced digests with amplicons from aerobic actinomycetes that comprised four general categories: (i) enzymes for which there were no recognition sites included *Bsa*WI, *Bsr*BI, *Bss*HII, *Bst*EII, *Eag*I, *Fse*I, *Nci*I, *Nhe*I, *Not*I, *Pvu*II, and *Stu*I; (ii) enzymes that produced generally large-band RFLP patterns with considerable intraspecies polymorphism included *Ban*II, *Nar*I, and *Xho*I; (iii) enzymes that gave two- to three-band RFLP patterns that were the same for multiple species included *Aat*II, *Bgl*I, *Bst*NI, and *Fsp*I (*Avi*II); and (iv) two- to four-band RFLP pattern-producing enzymes that enabled differentiation of several but not all species included *Aci*I, *Ban*I, *Bsp*1286I, *Hae*II, *Hae*III, *Hha*I (*Cfo*I), *Nae*I, and *Rsa*I. The enzymes in the last group exhibited intraspecies polymorphism that rendered them unacceptable for general use. Often, as many as eight endonucleases, predominantly those in the fourth category, would clearly differentiate two or three species or taxa from one another, a result similar to observations reported previously (28). As reported for *Nocardia* (21), none of the amplicons from the 64 nonnocardiae aerobic actinomycete isolates contained *Bst*EII recognition sites. Restriction fragment sizes remained consistent within each taxonomic group, as in prior studies (21, 22), and are expressed to the nearest 5 bp, as recommended by Telenti et al. (23).

The first five one- to three-band *Msp*I-derived RFLP patterns (Fig. 1) previously obtained from isolates of the *N. asteroides* complex types I, II, IV, and VI, *N. brasiliensis*, *N. brevicatena*, *N. carnea*, *N. farcinica*, *N. otitidiscaviarum*, and *N. transvalensis* (21) were also obtained from 31 of 64 (48%) isolates of other aerobic actinomycetes. These patterns included 10 of 11 *A. madurae* isolates, 2 of 12 *G. bronchialis*

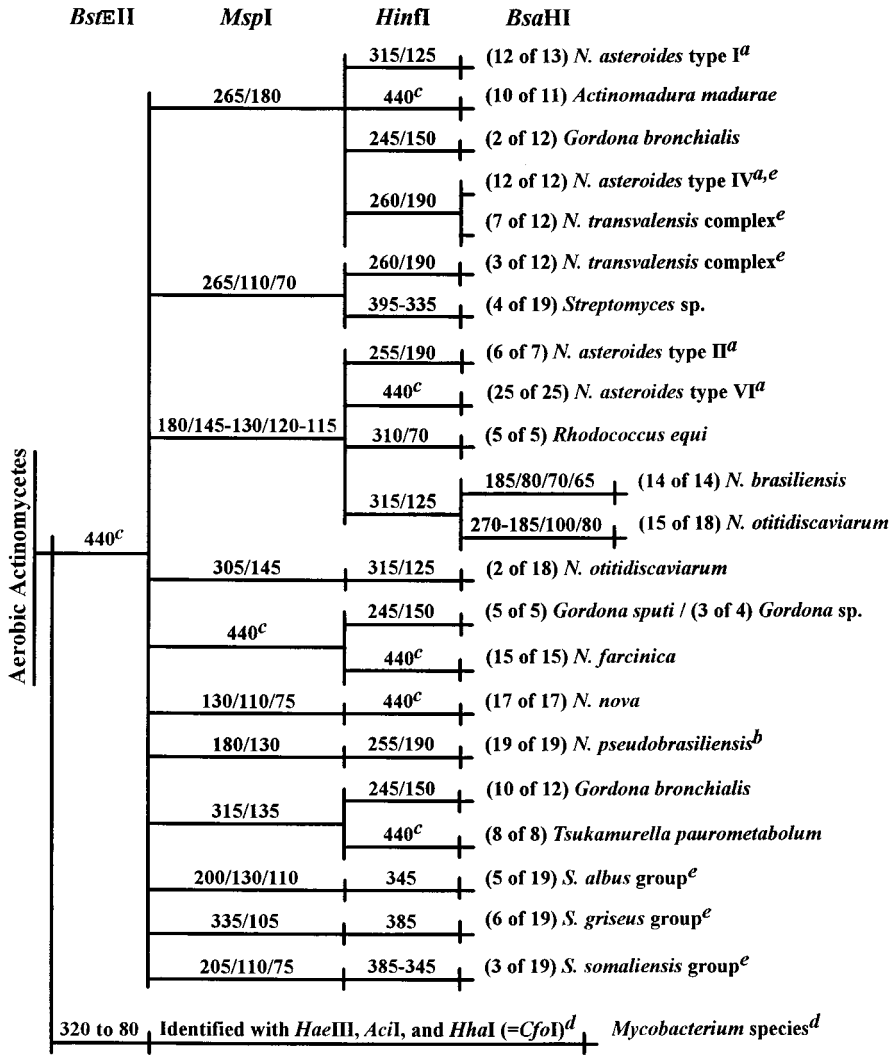

FIG. 1. Practical schematic for the identification of species and subgroups of aerobic actinomycetes by restriction endonuclease analysis of a PCR-amplified 65-kDa HSP gene sequence. RFLP band values are expressed as the number of nucleotide base pairs rounded to the nearest 5 bp, as described in the text. *a*, antimicrobial susceptibility (antibiogram) pattern types within the *N. asteroides* complex described by Wallace et al. (31); *b*, described by Wallace et al. (28) and Ruimy et al. (18); *c*, no recognition sites present for *Bst*EII, *Msp*I, or *Hin*fI; *d*, described by Telenti et al. (23) and Steingrube et al. (22); *e*, see text for definitions of the *N. transvalensis* complex (33) and *Streptomyces* groups.

isolates, 5 of 19 *Streptomyces* sp. isolates (Fig. 2A, lanes 1 to 4), 5 of 5 *R. equi* isolates, 5 of 5 *G. sputi* isolates, and 3 of 4 *Gordona* sp. isolates. The remaining 14 of 19 *Streptomyces* isolates exhibited three RFLP band patterns with *Msp*I, composed of two to four bands, that set them apart from all other aerobic actinomycetes (Fig. 1; Fig. 2A, lanes 7 to 9; and Fig. 2B). Each of these *Msp*I-defined RFLP band pattern groups of clinical *Streptomyces* isolates included one or two ATCC strains that were identified to the species level. These groupings of clinical isolates were therefore designated in Fig. 1 according to the species name of the ATCC strain that matched each group, i.e., the *S. albus* group, the *S. griseus* group, and the *S. somaliensis* group. Ten of 12 isolates of *G. bronchialis* (Fig. 1 and Fig. 2C, lanes 1 to 3) and all 8 isolates of *T. paurometabolum* (Fig. 1 and Fig. 2C, lanes 4 to 6) exhibited a unique two-band RFLP pattern of 315 and 135 bp with *Msp*I. The unique *Msp*I-derived RFLP band patterns exhibited by 2 of 18 isolates of *N. otitidiscaviarum* and all isolates of *N. nova* and *N. pseudobrasiliensis* (18) (formerly the unnamed new taxon [28]) were the same as those described previously (21) and were not shared with any other aerobic actinomycete species or taxon.

Some or all isolates belonging to six taxa, including 2 of 18 *N. otitidiscaviarum* isolates, 17 of 17 *N. nova* isolates, 19 of 19 *N. pseudobrasiliensis* isolates (18, 28), and 14 of 19 *Streptomyces* isolates, including *S. albus*, *S. griseus*, and *S. somaliensis*, could be identified by their RFLP band patterns with *Msp*I alone. Of the remaining 164 isolates, 161 were divided into the five distinct *Msp*I-derived pattern groups (Fig. 1).

Amplicon digestions with *Hin*fI eliminated the considerable intraspecies polymorphism noted previously when *Bsa*HI was used with *Nocardia* species (21). *Hin*fI produced single RFLP band patterns from amplicons of isolates comprising 15 of 18 taxa. As illustrated in Fig. 1, *Hin*fI used in conjunction with *Msp*I enabled complete differentiation of 14 of the 18 clinical species and taxa of aerobic actinomycetes (e.g., *G. bronchialis* and *T. paurometabolum* in Fig. 2C, lanes 9 to 11 and 12 to 14, respectively). *Hin*fI-generated RFLP band patterns divided the isolates of the remaining four taxa into two separate pairings:

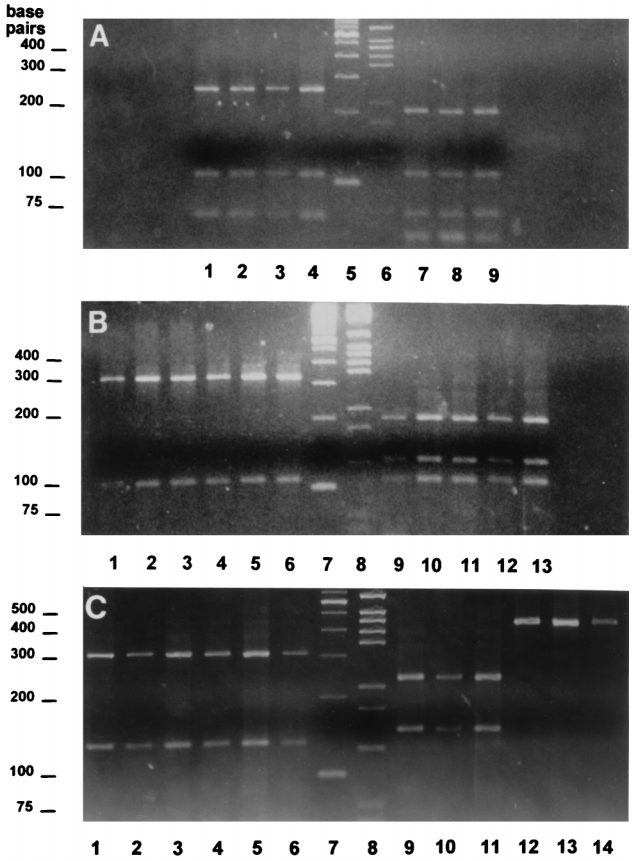

FIG. 2. RFLP band patterns from *Msp*I and *Hin*fI digests of PCR-amplified sequences of the 65-kDa HSP gene. (A) *Msp*I digests. Lanes 1 to 4, *Streptomyces* sp. isolates As 32, As 41, As 103, and As 105, respectively; lanes 5 and 6, size markers (100-bp and pGEM-bp ladders, respectively); lanes 7 to 9, *S. somaliensis* group isolates As 48, As 49, and ATCC 33201, respectively. (B) *Msp*I digests. Lanes 1 to 6, *S. griseus* group isolates As 44, As 85, As 88, As 101, ATCC 10137, and ATCC 23345, respectively; lanes 7 and 8, size markers (100-bp and pGEM-bp ladders, respectively); lanes 9 to 13, *S. albus* group isolates As 5, As 42, As 86, As 104, and ATCC 3004, respectively. (C) Lanes 1 to 3, *Msp*I digests from *G. bronchialis* As 87, Mo 280, and ATCC 25592, respectively; lanes 4 to 6, *Msp*I digests from *T. paurometabolum* Mb 228, ATCC 8368, and ATCC 25938, respectively; lanes 7 and 8, size markers (100-bp and pGEM-bp ladders, respectively); lanes 9 to 11, *Hin*fI digests from *G. bronchialis* As 87, Mo 280, and ATCC 25592, respectively; lanes 12 to 14, *Hin*fI digests from *T. paurometabolum* Mb 228, ATCC 8368, and ATCC 25938, respectively.

(i) *N. brasiliensis* and *N. otitidiscaviarum* and (ii) *N. asteroides* type IV and 7 of 12 isolates of *N. transvalensis*, as shown in Fig. 1.

Use of *Bsa*HI as the third restriction endonuclease in the identification system provided complete differentiation of one of the two paired taxa, namely, *N. brasiliensis* and *N. otitidiscaviarum* (Fig. 1). However, *Bsa*HI separated only 3 of 12 (25%) *N. transvalensis* isolates from those of *N. asteroides* type IV, as reported previously (21). Intraspecies polymorphism in *Bsa*HI-derived RFLP band patterns was observed with isolates comprising 10 of the 18 taxa in the present study. Although *Bsa*HI produced multiple RFLP band patterns from isolates of *N. otitidiscaviarum*, only a single pattern was produced from those of *N. brasiliensis*. Despite this heterogeneity, isolates of *N. otitidiscaviarum* were easily identified by the unique characteristic of being the only *Nocardia* taxon that exhibited RFLP band patterns containing two bands of ≥100 bp, as reported previously (21) (Fig. 1).

Compared with *Bsa*HI, intraspecies polymorphism was much less prevalent with *Msp*I and *Hin*fI. *Msp*I produced multiple patterns from four taxa: *N. asteroides* type VI, *N. transvalensis*, *N. otitidiscaviarum*, and *G. bronchialis*. With *Hin*fI polymorphism occurred only with isolates of the genus *Streptomyces* (Fig. 1).

Amplicons from all five isolates of *G. sputi* and three of four isolates of *Gordona* sp. exhibited no *Msp*I recognition sites and a two-band RFLP pattern of 245 and 150 bp with *Hin*fI that was unique for the genus *Gordona* (Fig. 1 and Fig. 2C, lanes 9 to 11). *Msp*I and *Hin*fI RFLP band patterns readily separated *G. sputi* and *G. bronchialis* (Fig. 1).

Overall, 182 of 188 (96.8%) clinical isolates that were clustered biochemically and/or by antibiograms exhibited distinctive species- or taxon-specific RFLP band patterns. The six exceptions to the common groupings presented in Fig. 1 included (i) 1 of 4 *Gordona* sp. isolates that exhibited an *Msp*I-derived pattern that resembled that of *N. brasiliensis*, but that produced the genus-specific 245- and 150-bp RFLP band pattern with *Hin*fI shown in Fig. 1 and Fig. 2C, lanes 9 to 11; (ii) 1 of 19 isolates of *Streptomyces* that lacked *Hin*fI recognition sites; (iii) 1 of 13 *N. asteroides* type I isolates that exhibited a 90-bp band instead of the common 125-bp band with *Hin*fI (Fig. 1); (iv) 1 of 7 *N. asteroides* type II isolates that lacked *Hin*fI recognition sites; and (v) 2 of 12 isolates of *N. transvalensis* that exhibited unique patterns with *Msp*I, but that produced the common band pattern with *Hin*fI. Two of 28 reference strains gave unique RFLP patterns, namely, (i) 1 of 3 strains of *A. madurae* (ATCC 13724) exhibited an *N. pseudobrasiliensis*-type pattern with *Msp*I, but showed the common 440-bp band pattern with *Hin*fI, and (ii) the type strain of *N. otitidiscaviarum* (ATCC 14629) exhibited a unique *Bsa*HI pattern of 150-, 115-, 70- and 65-bp bands, but contained two bands of ≥100 bp that characterized the species.

DISCUSSION

A previous report from this laboratory (UTHCT) of a PCR-RFLP identification protocol with the same 439-bp DNA segment used for the identification of *Mycobacterium* species (22, 23) showed *Msp*I and *Bsa*HI to provide nearly complete differentiation of all species and taxa of *Nocardia* (21). In the present study differentiation of other aerobic actinomycete species and taxa as well as some *Nocardia* taxa with these two endonucleases was found to be incomplete. Additionally, intraspecies polymorphism was a problem with *Bsa*HI-derived RFLP patterns. *Bsa*HI produced intraspecies polymorphism with 4 of 12 taxa of *Nocardia* (21) and with 10 of the 18 taxa in the current study. Multiple RFLP band patterns from several species in the differentiation schema tended to complicate species identification. The substitution of *Hin*fI for *Bsa*HI produced an identification system that completely differentiated 14 of the 18 taxa in this study. Limited use of *Bsa*HI in the present identification protocol resulted in intraspecies polymorphism only with isolates of *N. otitidiscaviarum*.

Of the four taxa that were not separated with *Msp*I and *Hin*fI, *Bsa*HI was effective in differentiating isolates of *N. brasiliensis* and *N. otitidiscaviarum*, but separated only 40% of the *N. transvalensis* isolates from those of the *N. asteroides* complex type IV. Isolates belonging to these latter two taxa were resistant to amikacin, a unique characteristic among nocardiae (21, 31). A preliminary report of the results of an ongoing investigation into the relationship between these two taxa has recommended that they be regarded collectively as the *N. transvalensis* complex (33). This complex was composed of three biochemically distinct subgroups, including *N. transvalensis* iso-

lates and *N. asteroides* complex type IV isolates, that were differentiated by PCR-RFLP analysis.

Although previously examined reference (ATCC) strains of *Nocardia brevicatena* and *Nocardia carnea* (21) also exhibited RFLP patterns with *Msp*I and *Hin*fI that were the same as those for the *N. asteroides* complex type VI and the *N. asteroides* complex type II, respectively, they were readily differentiated by their *Bsa*HI-derived patterns. These latter two species were omitted from the present study due to the unavailability of clinical isolates for comparison.

The intraspecies polymorphism observed in RFLP patterns from *Bsa*HI digests of *N. otitidiscaviarum* amplicons, the 36% (10 of 28 isolates) failure rate of PCR amplification with TB11 and TB12 primers, and the presence of two bands greater than 100 bp in *Bsa*HI-derived RFLP patterns that is unique among *Nocardia* isolates suggested that *N. otitidiscaviarum* may be quite different from other *Nocardia*. Interestingly, Chun and Goodfellow (7) reported that two sublines were recognizable within *Nocardia* on the basis of sequencing differences in the 16S rRNA gene. One of the sublines consisted of *N. asteroides* and related species, while the other was typified by *N. otitidiscaviarum*.

*Msp*I produced multiple RFLP band patterns from amplicons of *G. bronchialis*, the *N. asteroides* complex type VI, *N. otitidiscaviarum*, *N. transvalensis*, and the genus *Streptomyces*, while *Hin*fI did so only with the genus *Streptomyces*. Intraspecies polymorphism with *Msp*I was limited to two to three patterns, as opposed to those with *Bsa*HI and *Hae*III, which often produced up to five and six different patterns within individual species.

The multiple *Msp*I-derived RFLP patterns observed with *Streptomyces* isolates may prove to be associated with species differences, as indicated by the clustering of clinical isolates into pattern groups that included separate species represented by ATCC reference strains. With more than 3,000 individual species now recognized (2), *Streptomyces* isolates are customarily identified clinically to the genus level only and therefore were not differentiated further for the purposes of this study. *Msp*I-derived RFLP patterns alone were sufficient for separating 74% (14 of 19) of the *Streptomyces* isolates from all other aerobic actinomycetes. A more detailed study of a larger collection of clinical isolates of *Streptomyces* will be required in order to determine the degree to which the observed RFLP pattern heterogeneity is related to species differences or intraspecies polymorphism. *Hin*fI produced very large fragments of 335 to 395 bp from 18 of 19 isolates of *Streptomyces* that further facilitated differentiation. These very large RFLP bands were unique to *Streptomyces* isolates and readily distinguished them from isolates of all other aerobic actinomycete species and taxa.

The lack of *Bst*EII recognition sites in the amplified 65-kDa HSP gene sequence from all 216 isolates of aerobic actinomycetes in this study provided a rapid molecular technique for differentiating them from mycobacteria. As reported previously, amplicons from the majority of *Mycobacterium* species, including all pathogenic rapidly growing species and taxa, have one or more *Bst*EII recognition sites (22, 23).

As pointed out above, biochemical identification of clinical aerobic actinomycete isolates, in addition to being laborious and time-consuming, is not always diagnostic and may produce results that do not match typical criteria or those for designated type strains. Of the 21 *Gordona* isolates examined in this study, biochemical identification based on the carbohydrate utilization reactions described by Tsukamura (26) indicated that 12 isolates were *G. bronchialis*, 5 isolates were *G. sputi*, and 4 isolates did not match any identifiable pattern and were designated *Gordona* spp. PCR-RFLP analysis with *Msp*I and *Hin*fI produced RFLP band patterns for three of these four isolates that were identical to those exhibited by the five *G. sputi* isolates, thereby providing a conclusive identification that was not otherwise possible by biochemical methods. These distinctive RFLP band patterns also lended support to the validity of the species *G. sputi* proposed by Tsukamura in 1978 (26).

PCR amplification with two primers, TB11 and TB12, followed by restriction endonuclease analysis with five enzymes (*Bst*EII, *Hae*III, *Msp*I, *Hin*fI, and *Bsa*HI) has been used for the differentiation of 50 commonly encountered pathogenic species and taxa of aerobic actinomycetes comprising the genera *Mycobacterium* (22, 23), *Nocardia* (21, 28), *Actinomadura*, *Gordona*, *Rhodococcus*, *Tsukamurella*, and *Streptomyces*. This PCR-RFLP methodology distinguished clinical isolates of aerobic actinomycetes with 96.8% accuracy. Current ongoing studies have indicated that this system can be readily and economically implemented for routine clinical use. Identification of clinical isolates has been accomplished within 24 to 48 h of receipt of pure cultures in the UTHCT laboratory.

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